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The effect of transglutaminase on reconstituted skim milks at alkaline pH

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ABSTRACT

The effect of transglutaminase (TG) treatment on the changes in the physico-chemical properties of reconstituted skim milk (10% w/w) under alkaline conditions (pH ~6.7 to ~10) was investigated. The casein micelle hydrodynamic radius, the distribution of proteins between the serum and micelles, and the milk viscosity differed between milk treated with and without TG under alkalinisation. When milks were alkalinised from pH ~6.7 to ~8, both TG-untreated and TG-treated casein micelles swelled due to the increase in electrostatic repulsions between the negatively charged casein molecules. In milk without TG, the swelling of the micelles resulted in their progressive dissociation, while in the presence of TG-induced crosslinks, most caseins were maintained in their micellar form. When the pH increased from ~8 to ~10, TG-treated casein micelles resisted complete micellar disruption and continued to swell, whereas TG-untreated casein micelles disintegrated into smaller particles, with a marked increase in the soluble casein concentration. Under alkalinisation, only the α-lactalbumin present in the serum of TG treated milk was resistant to denaturation and aggregation. In response to all these micellar and serum changes, the viscosity increased for both TG untreated and treated milks. A simple qualitative model taking into account the extent of casein micelle dissociation and the size of the casein micelles was proposed to describe the behaviour of the viscosity as a function of pH. TG crosslinking of skim milk did not affect the partition of calcium, inorganic phosphorus, magnesium and potassium; and milk alkalinisation did not result in new calcium-phosphate phases.

1. Introduction

Skim milk is a suspension in which casein micelles are dispersed in a serum that predominately comprises water, lactose, whey proteins and a small quantity of salts, enzymes and vitamins (Fox & McSweeney, 1998; Holt, de Kruif, Tuinier, & Timmins, 2003; de Kruif, 1998). Casein micelles, with diameter of ~100–600 nm, are polydisperse amorphous aggregates consisting of mainly caseins (α_{s1}-, α_{s2}-, β- and κ-caseins) and the sequestered nanoclusters of amorphous calcium phosphates (Holt, Carver, Ecroyd, & Thorn, 2013). The caseins in the micelles are linked together by non-covalent interactions through long exon-encoded proline and glutamine rich sequences (Thorn, Ecroyd, Carver, & Holt, 2015). Amorphous calcium phosphate nanoclusters are tightly bound to short exon-encoded sequences called phosphate centre (PC) sequences in α_{s1}-, α_{s2}-and β-caseins. κ-caseins have the hydrophilic C-termini protruding out to the surface, forming the outer region of the micelles, providing steric repulsion and limiting the growth of the micelles (Holt & Carver, 2012). When a milk system is altered by a change in pH, ionic strength, temperature, high pressure or by the addition of chelatants, the structure of micelles is affected (Augustin & Clarke, 1991; Gaucheron, 2005).

Alkalinisation of milk results in changes in the physical properties of the casein micelle, such as the destabilisation and disruption of casein micelles (Ahmad, Piot, Rousseau, Grongnet, & Gaucheron, 2009; Hemar, Law, Horne, & Leaver, 2000; Horne, 1998; Madadlou, Mousavi, Emam-Djomeh, Sheehan, & Ehsani, 2009; Odagiri & Nickerson, 1965; Plomley, Higgins, & Hayes, 1951; Rose, 1968; Vaia, Smiddy, Kelly, & Huppertz, 2006; van Dijk, 1992). Increasing the stability and structural integrity of casein micelles may positively impact the overall functional properties of milk. Transglutaminase (TG), known to easily cross-link the different caseins in the micelle (Smiddy, Martin, Kelly, de Kruif, & Huppertz, 2006; de Kruif & Holt, 2003a), enhances the stability of milk against heat-induced coagulation by reducing the extent of heat-induced dissociation of κ-caseins on the micellar surface (O'Connell &

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Fox, 2003; O'Sullivan, Kelly, & Fox, 2002a, 2001). Other works reported that the enzymatic cross-linking action of the TG could increase the stability of casein micelles after the addition of urea, sodium dodecyl sulfate, or calcium-chelating agents (de Kruif, Tuinier, Holt, Timmins, & Rollema, 2002; O'Sullivan, Kelly, & Fox, 2002b; Smiddy et al., 2006), removal of calcium phosphate nanoclusters (Moon, Hong, Huppertz, Fox, & Kelly, 2009), in the presence of ethanol (O'Connell, Kelly, Auty, Fox, & de Kruif, 2001, b; Smiddy et al., 2006; Zadow, 1993), or under high hydrostatic pressure (O'Sullivan et al., 2002b).

TG is a protein-glutamine γ-glutamyltransferase (EC 2.3.2.13) (Gauche, Vieira, Ogliari, & Bordignon-Luiz, 2008; Ikura, Kometani, Yoshikawa, Sasaki, & Chiba, 1980; Lauber, Henle, & Klostermeyer, 2000; Law, 2010; Zhu, Rinzema, Tramper, & Bol, 1995). It catalyses acyl-transfer reactions by linking the γ-carboxyamide groups of peptide-bound glutamine residues, which act as acyl donors, with the ε-amino group of peptide-bound lysine residues acting as acyl acceptors. The resulting covalent bond formed between protein substrates is known as an ε-(γ-glutamyl)-lysine isopeptide bond, in which polypeptide protein chains are polymerised and crosslinked (Nonaka et al., 1989; de Jong & Koppelman, 2002). In milk, κ-caseins are most susceptible to TG crosslinking then to a lesser extent, β-caseins, and α_s-caseins are the most poorly accessible, due to the location within the casein micelles (Smiddy et al., 2006; de Kruif & Holt, 2003a). The accessibility of whey proteins to TG crosslinking is low, as compared to caseins, because glutamine and lysine residues are buried inside the globular structures of the whey proteins (O'Sullivan et al., 2002b). When other ε-amino groups act as acyl acceptors, the amine incorporation reaction occurs (Zhu et al., 1995). In the absence of primary amines, the deamidation reaction occurs as water acts as the acyl acceptor (Arrizubieta, 2007).

To the best of our knowledge, the capability of TG to affect the impact of alkalinisation in milk has not been investigated. This study explores the influence of TG on milk during the alkalinisation process (from ~pH 6.7 to ~ pH 10), specifically to investigate the changes in its physico-chemical properties in comparison with TG-untreated milk. In the present paper, milk viscosity, casein micelle particle size, partition of proteins between the micellar phase and the serum, as well as the changes in the mineral partition between the protein phase and the serum, and the calcium phosphate phases present in the casein micelles, are investigated. The findings of this study might contribute to the fundamental knowledge on casein micelle structure which is still not fully known.

2. Materials and methods

2.1. Materials

Low heat skim milk powder was generously supplied by Westland Co-Operative Dairy Company Limited (Hokitika, New Zealand). The chemical composition of the skim milk powder as provided by the manufacturer was as follows: 33.5% protein; 7.8% minerals and 3.7% moisture. TG is a commercial enzyme preparation (TG-BW-MH) gifted by Ajinomoto Berhad (Kuala Lumpur, Malaysia) and was used without any further purification. The specific TG activity was determined to be 634 ± 2 U/g according to the hydroxamate method of Folk and Cole (1966). The protein standards (α_s-casein (α_s-CN), β-casein (β-CN), κ-casein (κ-CN), α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg)) used were obtained from Sigma-Aldrich Co., (Missouri, USA). All the chemicals used were of analytical grade, with the exception of acetonitrile and trifluoroacetic acid which were HPLC grade. Milli-Q water (resistivity at 18.2 MΩ cm) was used with 0.02% (w/w) sodium azide added as a preservative to prevent bacterial growth.

2.2. Preparation of milk samples

Stock reconstituted skim milk (16.6% w/w milk solids) was prepared by gently mixing low heat skim milk powder with Milli-Q water for 2 h to ensure thorough dispersion. The stirred milks were stored at 4 °C overnight to ensure full hydration. They were equilibrated at room temperature then divided into two equal fractions. One fraction was treated with TG solution (final enzyme/substrate weight ratio of 0.15), and the other fraction, termed TG-untreated, served as the control. The pH of the milk fractions (with and without TG) was adjusted to 6.70 by the addition of 1 M HCl or 1 M NaOH. Both milk fractions (with and without TG) had milk concentrations brought to 15% (w/w) milk solids and were incubated at 30 °C for 15 h. After incubation, the milks were heat treated at 70 °C for 15 min to inactivate the TG. Milks were divided into equal portions of 40 g and then alkalinised to pH values between ~6.7 and ~10 by the addition of different amounts of 0.1 M NaOH. Milli-Q water was added to each sample up to a total mass of 60 g, diluting each sample from 15% (w/w) total milk solids to 10% (w/w). Samples were stored overnight at 4 °C to ensure full hydration and then further equilibrated for 3 h to room temperature prior to analysis.

2.3. Isolation of milk serum fractions and collection of ultrafiltrated sera

Alkalinised milk samples were placed in 30 mL Nalgene centrifuge tubes (Oak Ridge Style 3119, Thermo Scientific, New York, USA) and centrifuged at 38,000 g for 60 min at 20 °C in a Sorvall RC 6 Plus Superspeed Centrifuge (North Carolina, USA) with a Fiberlite fixed angle rotor F21-8x50y. The supernatant or milk serum was carefully removed from the pellet. This serum fraction was used as is or used for further collection of ultrafiltrated sera. The ultrafiltrates were obtained by centrifuging the centrifuged supernatants in Vivaspin Turbo 15 tubes containing polyethersulfone membranes with a molecular weight cut-off of 10 kDa (Sartorius Stedim Biotech GmbH, Goettingen, Germany) at 1500 g for 60 min at 25 °C, using the Heraeus Centrifuge (Labofuge 400, Thermo Scientific, Hanau, Germany).

2.4. Particle sizing

The particle size of casein micelles was determined by dynamic light scattering, performed on the Malvern ZSP Nano Zetasizer (Malvern Instruments, Worcestershire, UK) fitted with a 5 mW 633 nm helium-neon laser. Milk samples (10 μL) were diluted in 1.5 mL of their ultrafiltrates in cuvettes with a pathlength of 1 cm. Ten measurements were taken for each sample and all measurements were performed at 25 °C. A refractive index of 1.34, measured on the ultrafiltrate using a refractometer (Atago Co., Ltd., Tokyo, Japan), and a viscosity of 0.89 mPa s, measured by a capillary viscometer (Schott, SI Analytics, Mainz, Germany), were used for casein micelles size determination.

2.5. Characterisation and quantification of milk proteins

Reversed phase-high performance liquid chromatography (RP-HPLC) was used to characterise and quantify the casein and whey contents in milk and serum samples. The pre-treatment of samples for casein and whey proteins was adapted from Bobe, Beitz, Freeman, and Lindberg (1998) and Jean, Renan, Famelart, and Guyomarc'h (2006). For the separation of caseins, milk samples and centrifuged supernatants (200 μL) were treated with a mixture (200 μL) containing 1:1:1 ratio (v:v:v) of 0.1 M BisTris buffer (pH 6.8), 6 M guanidine hydrochloride and 5.37 mM trisodium citrate dihydrate, followed by the addition of 5 μL of 19.5 mM dithiothreitol (pH 7). These mixtures were stirred for 10 s using a vortex mixer and left to stand for 1 h at room